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Infectious RNA Transcripts from Full-Length Dengue Virus Type 2 cDNA Clones Made in Yeast

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The dengue virus type 2 genomic RNA was amplified by reverse transcription-PCR and cloned as four cDNA fragments. We could not assemble these four fragments into full-length cDNA in *Escherichia coli*. The full-length dengue virus cDNA was constructed by homologous recombination in yeast, either as part of a yeast artificial chromosome or in a yeast-*E. coli* shuttle vector. Full-length cDNA clones were propagated once in *E. coli* to prepare useful quantities of DNA. In vitro transcription of these clones produced full-length RNA transcripts. Introduction of these transcripts into LLC-MK_2 cells produced typical dengue infection, as judged by cytopathic effects and indirect immunofluorescence. Infectivity was sensitive to RNase digestion and was dependent on the presence of cap analog in the transcription reaction mixture. Virus in the medium was passaged on C6-36 cells to produce stocks, and these stocks had titers and plaque morphologies similar to those of the parental dengue virus type 2. Intracellular dengue virus RNA from cells infected with transcript-derived virus contained an introduced *Bst*EII site, proving that infectivity was derived from RNA transcripts and not from contamination with parental dengue virus. Transcript-derived virus was comparable to dengue virus type 2 for growth and protein expression in tissue culture cells. Sequence analysis of the dengue virus cDNA in one full-length clone revealed only one unexpected silent mutation. By using yeast technology, it will be easy to introduce specific mutations into the dengue virus cDNA, allowing analysis of the virus phenotype in cells transfected with mutant transcripts.

The *Flavivirus* genus comprises about 70 viruses, including some important human pathogens such as Japanese encephalitis virus (JE), yellow fever virus (YF), the tick-borne encephalitis viruses, and dengue virus (DEN), which are transmitted to humans by mosquito or tick vectors. DEN infection is probably the most widespread vector-borne human viral infection in the world, with an estimated incidence of up to 100 million cases each year (21). The predominant disease caused by DEN is dengue fever, a self-limited febrile illness, often accompanied by minor hemorrhagic manifestations. Infrequently, DEN infection leads to the much more serious dengue hemorrhagic fever/shock syndrome, which can have case fatality rates as high as 20% (21).

The flavivirus genome is a single-stranded, positive-sense RNA molecule of 10.5 to 11 kb, containing a single open reading frame constituting roughly 95% of the genome. Upon infection, the viral RNA is translated into a polyprotein of about 3,400 amino acids that is processed co- and posttranslationally into 10 gene products: the three structural proteins core protein (C), premembrane protein (prM), and envelope protein (E) and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (4, 9). Introduction of flavivirus genomic RNA into susceptible hosts or cells in culture leads to a productive virus infection (1, 27). As was first shown for brome mosaic virus and human rhinovirus, clones containing the full-length cDNA of a positive-strand RNA virus genome can be constructed so that in vitro transcription produces RNA transcripts which are replicas of the genomic RNA, and introduction of these transcripts into susceptible hosts or cells in culture results in a productive virus infection (2, 20). Such an "infectious clone" is a valuable tool for genetic analysis, as mutations can be introduced into any region of the genome, and the effect on the phenotype of the virus can be analyzed after transfection of mutant transcripts into cells.

The first infectious clone of a flavivirus was obtained for YF in 1989 (22). Subsequently, infectious clones have been described for DEN type 4 (DEN4) (18), JE (28), Kunjin virus (17), and DEN2 (16). However, obtaining and working with these flavivirus infectious clones have not been easy, due to the apparent instability of cloned full-length flavivirus sequences in *Escherichia coli*. Indeed, for YF, JE, and DEN2, the infectious clone is actually two clones, each containing roughly half the genomic cDNA. Two-piece construction was necessary in these cases because full-length cDNA clones capable of producing infectious transcripts were never obtained. To use these two-clone systems, the two pieces of cDNA are ligated together and directly transcribed, and the mixture of transcription products is introduced into cells. For DEN4 and Kunjin virus, one-piece infectious clones were obtained in the low-copy-number vector pBR322, at the expense of high DNA yield. Even so, the DEN4 clone could be described as metastable, as some bacterial colonies harbor rearranged versions of this plasmid, with large deletions or insertions in the DEN sequences (7). Recently, one group has finessed the flavivirus full-length clone instability problem completely, describing a method for making infectious transcripts of tick-borne encephalitis viruses without any cloning (11). In this method, half-genome-sized cDNA products were made by reverse transcription-PCR (RT-PCR), and then the two halves were ligated and directly transcribed.

DNA sequences which are unstable in *E. coli* can frequently be stably cloned in eukaryotic systems. The yeast *Saccharomy-*

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ces cerevisiae is a popular eukaryotic host for these purposes. Two common strategies for cloning DNA in yeast are the assembly of yeast artificial chromosomes (YACs) and the use of yeast-*E. coli* shuttle vectors. We sought to make a full-length infectious clone of DEN2, New Guinea C (NGC) strain, since such a clone would be simpler to use than the two-piece DEN2 infectious clone. This report describes how we successfully overcame the *E. coli* instability problem by assembling the full-length DEN2 cDNA in yeast.

MATERIALS AND METHODS

Cells and viruses. Monkey LLC-MK_2 cells were grown at 37°C in a humidified incubator under 5% CO_2 in growth medium consisting of Eagle's minimal essential medium (MEM) plus 10% fetal bovine serum (FBS) and 50 μg of gentamicin sulfate per ml. Mosquito C6-36 cells were grown at 30°C in the same medium supplemented with nonessential amino acids and sodium pyruvate plus 25 mM HEPES, pH 7.55.

Parental DEN2 NGC had been passaged in suckling mouse brain 38 times. This virus was passaged several times in C6-36 cells, and virus stocks were stored frozen at −70°C.

Frozen competent *E. coli* strains DH5αF′IQ (used for routine subcloning) and STBL2 (used to propagate full-length clones) were purchased from Life Technologies Inc. (LTI).

S. cerevisiae YPH857 (26) was obtained from Forrest Spencer (Johns Hopkins University, Baltimore, Md.). The yeast was made competent by the lithium acetate procedure (26).

Virus titration. Virus titers were determined by plaquing on LLC-MK_2 cells in six-well dishes. Serial dilutions of virus were adsorbed for 1.5 h at room temperature, and the cells were overlaid with 6 ml of overlay medium (1× Earle's balanced salt solution without phenol red, 0.375% sodium bicarbonate, 0.5× MEM vitamins, and 0.5× MEM amino acids [all from LTI], 1% SeaKem ME agarose [FMC Bioproducts], and 10% FBS) and incubated at 37°C. On day 8, the wells were overlaid with 3 ml of staining medium (1% SeaKem ME agarose plus 0.396 mg of neutral red per ml in deionized water), and incubation at 37°C was continued. Plaques were counted the next day.

Preparation of RNA. To prepare viral RNA, DEN2 NGC was grown in C6-36 cells, and 35 ml of clarified medium was adjusted to 0.4 M NaCl–7% polyethylene glycol 8000 and incubated at 4°C overnight to precipitate the virus. The precipitate was collected by centrifugation, and RNA was prepared by one of two methods. In the first method, the virus pellet was resuspended in a small volume consisting of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS) and extracted twice with phenol and once with chloroform, and RNA was precipitated with ethanol and redissolved in 20 to 50 μl of RNase-free water. More recently, RNA has been prepared from the virus pellet by using a QIAamp HCV kit (Qiagen), resulting in a final sample volume of 50 μl. To prepare intracellular RNA, cells were harvested by scraping, and RNA was extracted from the cell pellet with a Qiagen RNeasy kit, leading to a final sample volume of 30 μl.

Primers. Nine primers were designed by using the published sequence of DEN2 NGC (14). Important features of primer SP1 include an *Xba*I site followed by 18 nucleotides (nt) encoding an SP6 RNA polymerase promoter and then 21 nt representing the 5′ end of DEN2. The 5′-terminal 7 nt of DEN2 NGC were not reported by Irie et al. (14), so the sequence AGTTGTT determined for DEN2 Jamaica was used (6). Transcription from the SP6 promoter in primer SP1 initiates with a G 1 nt upstream of the DEN2 5′-end sequence. Primers SP1.5B (antisense, DEN2 nt 3214 to 3183) and SP2.5B (sense, DEN2 nt 3184 to 3215) each introduce a silent C-to-T mutation at DEN nt 3198 that creates a unique *Bst*EII site. Primer SP2.5BL (sense, DEN2 nt 3163 to 3215) introduces the same mutation but includes more DEN2 sequence before the *Bst*EII site, to provide more overlap for homologous recombination in yeast. Primers SP2 (antisense, DEN2 nt 5484 to 5467), SP3 (sense, DEN2 nt 5389 to 5406), SP3.5 (antisense, DEN2 nt 7920 to 7894), and SP4.5 (sense, DEN2 nt 7708 to 7734) encode only DEN2 sequences and are located to take advantage of naturally occurring *Xho*I and *Stu*I sites. Finally, primer SP4 encodes a *Sac*I site immediately followed by the antisense 3′-terminal 40 nt of DEN2 (antisense, nt 10723 to 10684).

RT. Prior to RT reactions using viral RNA as a template, 10 μl of RNA with or without 100 ng of primer SP4 was incubated at 65°C for 3 min and then chilled on ice. RT reaction mixtures contained this heat-denatured RNA plus 0.5 mM (each) dATP, dCTP, dGTP, and dTTP; 10 mM dithiothreitol (DTT); 33 U of RNasin (Promega); and either 50 U of StrataScript reverse transcriptase (Stratagene) or 200 U of Superscript II reverse transcriptase (LTI) plus the corresponding buffer (1×), in a final volume of 25 or 50 μl. For intracellular RNAs, 6 μl of RNA plus 66 ng of primer SP3.5 in a volume of 8 μl was preheated and chilled as described above and then used as a template in an RT reaction using 0.312 mM (each) dATP, dCTP, dGTP, and dTTP; 10 mM DTT; 16.5 U of RNasin; and 50 U of StrataScript RT plus 1× buffer, in a final volume of 20 μl. All RT reaction mixtures were incubated at 42°C for 1.5 h and then stored frozen at −20°C.

PCR. PCRs were set up to amplify the DEN2 genome as four cDNA fragments: B (SP6 RNA polymerase promoter followed by DEN2 nt 1 to 3214), C (DEN2 nt 3184 to 5484), A (DEN2 nt 5389 to 7920), and D (DEN2 nt 7708 to 10723 plus a *Sac*I site). *Pfu* polymerase (Stratagene) was used because of its reported low error rate. The reaction mixture used to make cDNA fragment A contained 1 μl of RT product as a template; 165 ng each of primers SP3 and SP3.5; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 1× *Pfu* polymerase buffer 1; and 2.5 U of *Pfu* polymerase, in a total reaction volume of 100 μl. The reaction mixture was preheated to 94°C for 2 min and then subjected to 35 temperature cycles, each cycle being 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, followed by one final cycle in which the time at 72°C was increased to 10 min. The PCR mixture used to make fragment C contained primers SP2 and SP2.5B instead of SP3 and SP3.5 but was otherwise identical to the reaction mixture used to make cDNA A. To make the longer overlap version of cDNA fragment C, primer SP2.5BL replaced primer SP2.5B. The reaction mixture to make cDNA B was almost identical but used primers SP1 and SP1.5 and included 5% glycerol. The PCR mixture used for fragment D contained primers SP4 and SP4.5 and 10% dimethyl sulfoxide, and the reaction used 65°C instead of 55°C in the middle part of each temperature cycle. The template used in the PCRs to make cDNA fragments A, B, and C was RT product made from viral RNA in the absence of exogenous primer (see Results). The template used to make cDNA fragment D was RT product that had been made from viral RNA primed with SP4.

PCRs using RT products made from intracellular RNA as a template were also done. These reaction mixtures contained 4 μl of template; 165 ng each of primers SP2 and SP2.5 (sense, DEN2 nt 2250 to 2277); 0.35 mM (each) dATP, dCTP, dGTP, and dTTP; 2.5 U of Expand Polymerase (Boehringer-Mannheim); and 1× Expand buffer 1 which had been preadjusted to pH 9.2, in a final volume of 50 μl. The reaction mixtures were subjected to 35 temperature cycles, each cycle being 94°C for 10 s, 60°C for 30 s, and 68°C for 5 min.

Subcloning. Restriction enzymes and T4 DNA ligase were purchased from LTI or New England BioLabs (NEBL). The plasmid vector pGEM11Zf^+ (Promega) was modified by using mutagenic oligonucleotides to incorporate a *Stu*I site in place of the *Eco*RI site and a *Bst*EII site at the *Bam*HI site. cDNAs A, B, C, and D were digested with the appropriate pairs of restriction enzymes (*Xba*I/*Bst*EII for fragment B, *Bst*EII/*Xho*I for fragment C, *Xho*I/*Stu*I for fragment A, and *Stu*I/*Sac*I for fragment D) and then were cloned into similarly digested modified pGEM11Zf^+, generating clones pGEM A to D. Subsequently, by using the *Xho*I and *Stu*I sites, the A and D fragments were combined to make the right-half clone pGEM AD. Efforts to obtain the left-half clone pGEM BC failed. Subsequently, the B, C, and AD fragments were subcloned from pGEM into a version of the low-copy-number vector pCL1921 (19) with a modified polylinker, creating pCL B, pCL C, and pCL AD, respectively. Efforts to make the left-half clone pCL BC also failed.

Construction of full-length DEN2 cDNA in yeast. The method used to assemble the full-length DEN2 cDNA in a YAC is shown below (see Fig. 1). The *Not*I/*Xho*I fragment of pGEM B containing cDNA B was ligated to *Not*I/*Sal*I-digested pRML1 (26) to make pRML1 B. Next, an oligonucleotide was used to modify the *Sac*I site in pGEM AD to add a second *Bam*HI site. The resulting construct was digested with *Bam*HI, the fragment containing the right-half cDNA AD was subcloned into the *Bgl*II site of pRML2 (26), and a clone with the proper orientation was designated pRML2 AD. Both pRML1 B and pRML2 AD were linearized with *Cla*I, and the linearized DNAs (1.3 μg each) were mixed with 0.3 μg of the long overlap version of RT-PCR product C. The mixture was used to transform competent *S. cerevisiae* YPH857 to growth on solid medium lacking tryptophan and uracil ("dropout medium" [26]).

The approach used to assemble the full-length DEN2 cDNA in the shuttle vector pRS424 (5) is illustrated below (see Fig. 2). The *Not*I/*Xho*I fragment of pGEM B containing cDNA B was ligated to *Bsp*120I/*Xho*I-digested pRS424 to make pRS B. The right-half cDNA AD was subcloned as a *Sac*I/*Xho*I fragment of pCL AD into pRS424, creating pRS AD. Both pRS B and pRS AD were digested with *Xho*I and *Nsi*I, and the large fragments were ligated together to make pRS BAD. This clone was digested with *Xho*I, 6 μg of linear pRS BAD was mixed with 1 μg of the longer version of RT-PCR product C, and the mixture was used to transform competent YPH857 yeast to growth on solid tryptophan dropout medium.

Individual yeast colonies were screened by PCR (13), using primers which would amplify all or part of the C fragment. Positive clones were grown in 25 to 50 ml of liquid dropout medium, and yeast DNA mini-preps were performed by either of two methods as described elsewhere (23, 26). Yeast DNA was digested with appropriate restriction enzymes and analyzed by Southern blotting using random-primed DEN2 cDNA as a probe to confirm the structure of the DEN2 cDNA (24). The pRS424 vector harboring the full-length DEN2 cDNA was designated pRS BCAD, whereas the analogous YAC was referred to as the full-length YAC.

Purification of full-length clone DNA. To prepare full-length shuttle vector DNA, yeast DNA from clones harboring pRS BCAD was used to transform competent *E. coli* STBL2 cells to ampicillin resistance. Individual small colonies were usually picked directly into 50-ml cultures of Luria-Bertani broth containing 100 μg of ampicillin per ml and grown at 30°C for 2 to 3 days. Plasmid DNA was then purified with a Qiagen midi-prep kit. Occasionally, colonies were picked into 2-ml cultures and prepared by standard mini-prep procedures. The restric-

tion pattern of purified plasmid DNA was analyzed to confirm the structure of the DEN2 cDNA.

More steps were required to recover the DEN2 cDNA from the full-length YAC (see Fig. 1d). DNA prepared from yeast cultures harboring the full-length YAC was digested with *Xba*I, diluted, self-ligated, and used to transform STBL2 cells to ampicillin resistance. Colonies were picked and screened as described above, and the resulting clones containing full-length DEN2 cDNA were designated pRML2′ BCAD.

In vitro transcription of full-length RNA. Full-length clones were linearized with *Sac*I or with its neoschizomer *Ecl*136II, extracted with phenol-chloroform, ethanol precipitated, and redissolved in RNase-free water. Complete in vitro transcription reaction mixtures contained 1 to 2 μg of linearized DNA; 0.5 mM (each) ATP, CTP, and UTP; 0.1 mM GTP; 0.5 mM cap analog $m^7G(5')ppp(5')G$ (Boehringer-Mannheim or NEBL); 10 mM DTT; 40 U of RNasin (Promega); 30 U of SP6 RNA polymerase; and 1× SP6 RNA polymerase buffer (Promega, LTI, or NEBL) in a volume of 30 μl. The reaction mixtures were incubated at 40°C for 1 to 2 h, and 3 μl was removed for agarose gel electrophoresis analysis and the remainder was stored at −70°C. As determined by inspection of the agarose gels, the typical yield of RNA was approximately 5 μg. In control reaction mixtures in which the cap analog was omitted, the concentration of GTP was increased to 0.5 mM.

Introduction of RNA transcripts into cells. In most experiments, the RNA transcription reaction mixtures were thawed and used without further processing. For certain control experiments, 10-μl aliquots of the thawed transcription reaction mixtures were first digested with 0.01 mg of DNase-free RNase A or with 1 U of RNase-free RQ1 DNase (Promega) at 37°C for 1 h.

Initially, cells were transfected with RNA by using Lipofectin reagent (LTI). Typically, 5 to 10 μl of transcription reaction mixture was combined with 30 μl of Lipofectin in a 2-ml volume of MEM, and after a 10-min incubation at room temperature, the mixture was added to confluent LLC-MK_2 cells in one well of a six-well dish. After 4 to 6 h, the Lipofectin mixture was replaced with growth medium. As a positive control, 1 μl of DEN2 viral RNA was used instead of transcription reaction mixture. The cells were examined daily for cytopathic effects (CPE), which for DEN infection of these cells is recognized as hypertrophy of cytoplasmic vacuoles. Cells were periodically seeded onto Labtek chamber slides or glass coverslips for indirect immunofluorescence analysis (see below).

More recently, RNA has been introduced into LLC-MK_2 cells by electroporation. Approximately 2×10^6 cells in 0.3 ml of phosphate-buffered saline (PBS) (Digene) were mixed with 5 to 10 μl of transcription reaction mixture, or with 1 μl of DEN2 RNA, in an electroporation cuvette with a 0.4-cm gap. Following incubation on ice for 10 min, each cuvette was pulsed at 200 V, 950 μF, and the cells were resuspended in growth medium and plated onto one well of a six-well dish. The medium was replaced the next day. The cells were examined daily for CPE and occasionally seeded onto chamber slides or coverslips for immunofluorescence analysis (see below).

In experiments to compare the specific infectivities of transcripts and viral RNA, cells in PBS as described above were mixed with various dilutions of RNA and then pulsed at 160 V, 950 μF. In order to fully disperse the pulsed cells, they were resuspended with 0.7 ml of 0.05% trypsin–0.53 mM EDTA; then the cells were mixed with 10 ml of growth medium and incubated at room temperature for 10 to 15 min to recover. After the recovery period, the cells were pelleted, resuspended in growth medium, plated onto wells, and incubated at 37°C. After 4 h of incubation, the medium was removed and the cells were overlaid with agarose and subsequently stained with neutral red on day 8, as for a virus titration.

Indirect immunofluorescence to detect viral antigens. Cells growing on Labtek chamber slides or glass coverslips were rinsed in room-temperature PBS and then fixed in cold acetone for 10 min at −20°C, air dried, and rehydrated in PBS plus 2% normal goat serum at room temperature. Each coverslip was inverted on top of 30 μl of a 1:50 dilution of DEN2-specific hyperimmune mouse ascitic fluid (HMAF) (American Type Culture Collection) in PBS plus 2% normal goat serum. Each chamber was covered with 50 μl of a 1:100 dilution of HMAF. Samples were incubated at 37°C for 1 h in a humidified atmosphere and then rinsed four times in PBS. Samples were then similarly incubated with a 1:50 or 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Kirkegaard & Perry Laboratories) and rinsed four times in PBS. Coverslips were fixed to slides with Aqua-Mount (Lerner Laboratories), while several drops of Fluoroguard (Bio-Rad) were added to chamber slides. Samples were observed and photographed with a Leitz fluorescent microscope.

Radiolabeling and analysis of infected-cell proteins. LLC-MK_2 cells in duplicate wells of a six-well dish were mock infected or were infected at a multiplicity of infection (MOI) of 0.125 PFU/cell with parental DEN2 NGC or with transcript-derived virus 3-3. On day 3 postinfection, when CPE was detected, the cells were starved for 1 h in methionine-free MEM plus 2% FBS, and then one set of wells was labeled for 4 h with 0.75 ml of the same medium containing 150 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham) per ml while the other member of each set was similarly labeled for 21 h with labeling medium to which 5% of the normal concentration of methionine had been added. The labeling media were removed, the wells were rinsed in PBS, and the cells were lysed in situ with 0.5 ml of ice-cold radioimmunoprecipitation assay buffer (8). A 100-μl sample of each clarified lysate was immunoprecipitated with 5 μl of DEN2-specific HMAF, and the precipitates were collected on Pansorbin (Calbiochem) beads, washed, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described previously (10).

Growth curves. LLC-MK_2 cells in a six-well dish were infected with parental DEN2 NGC or with transcript-derived viruses at an MOI of 0.01 PFU/cell. C6-36 cells in T-25 flasks were similarly infected at an MOI of 0.0025 PFU/cell. Aliquots of the culture fluid were removed daily and stored frozen at −70°C. The titer of DEN2 virus in each sample was determined by titration on LLC-MK_2 cells.

DNA sequencing. All sequencing of plasmid DNAs was done by the dideoxy method using Sequenase 2.0 (Amersham) and oligonucleotide primers made in-house. Direct sequencing of RT-PCR products was either done similarly or done on an ABI model 377 DNA sequencer using an ABI Prism dye terminator cycle sequencing kit with *Taq* DNA polymerase FS (Perkin-Elmer).

Artwork for Fig. 3, 4, and 6. Photographs (Fig. 3 and 4) and an autoradiograph (Fig. 6) were scanned on a Scan-Jet IIc (Hewlett-Packard). The images were cropped and labeled by using Adobe Photoshop 3.0 and then printed on photographic paper.

RESULTS

RT-PCR and cloning of the DEN2 NGC genome as four cDNA fragments. Initially, our plan was to make cDNA fragments representing the entire DEN2 NGC genome by RT-PCR using *Pfu* polymerase and to then clone and assemble them into a full-length cDNA in *E. coli*. Primers were designed to allow RT-PCR amplification of DEN2 NGC as four cDNA fragments of 2.3 to 3.2 kb with unique restriction sites located at both ends of each cDNA, for use in cloning and assembly (see Materials and Methods). Primer SP1 also introduces an SP6 RNA polymerase promoter just upstream of the DEN2 sequence.

RNA purified from the culture media of DEN2 NGC-infected cells was used as a template for the four RT-PCRs described in Materials and Methods. Interestingly, for the reactions to make cDNA fragments A, B, and C, no exogenous primer was added during the RT step. The success of this approach was surprising, but not only did it work, it also resulted in cleaner PCR products than those obtained by using primed RT reaction mixtures. Direct PCR of viral RNA without an intervening RT step yielded no products. Presumably, therefore, either the viral RNA is contaminated with primers or the reverse transcriptase can initiate cDNA synthesis from the 3′-terminal hairpin structure (12). The cDNA products were digested with the appropriate restriction enzymes and cloned first in *E. coli* into the high-copy-number plasmid pGEM11Zf$^+$ and subsequently into the low-copy-number plasmid pCL1921. In theory, these clones could be used to assemble a clone containing full-length cDNA. However, in practice, efforts to make such full-length clones in *E. coli* failed.

Assembly of full-length cDNA clones in yeast. Having failed to assemble the four DEN cDNAs in bacteria, we decided to try to assemble them in yeast. Two strategies were employed for making a full-length DEN2 NGC cDNA clone in *S. cerevisiae*: construction of a YAC and use of the yeast-*E. coli* shuttle vector pRS424. Both strategies were successful, and they are discussed in turn below.

The method used to assemble the four cloned fragments, A to D, into full-length DEN2 cDNA in a YAC is shown in Fig. 1. First, pRML1 B and pRML2 AD were constructed in *E. coli* by cloning cDNA B into pRML1 and cloning the right-half clone AD into pRML2, oriented as shown in Fig. 1a and b. Plasmids pRML1 and pRML2 have sequences that permit replication and selection in *E. coli* (ori and Amp^r) and in *S. cerevisiae* (ARS and *TRP1* or *URA3*). Both vectors also contain a telomere, and pRML1 contains a centromere. Digestion of pRML1 B and pRML2 AD with *Cla*I made linear molecules with DEN sequences near one end and a telomere at the other end (Fig. 1d). Meanwhile, an RT-PCR using DEN2 NGC RNA as a template was done to make a slightly longer version

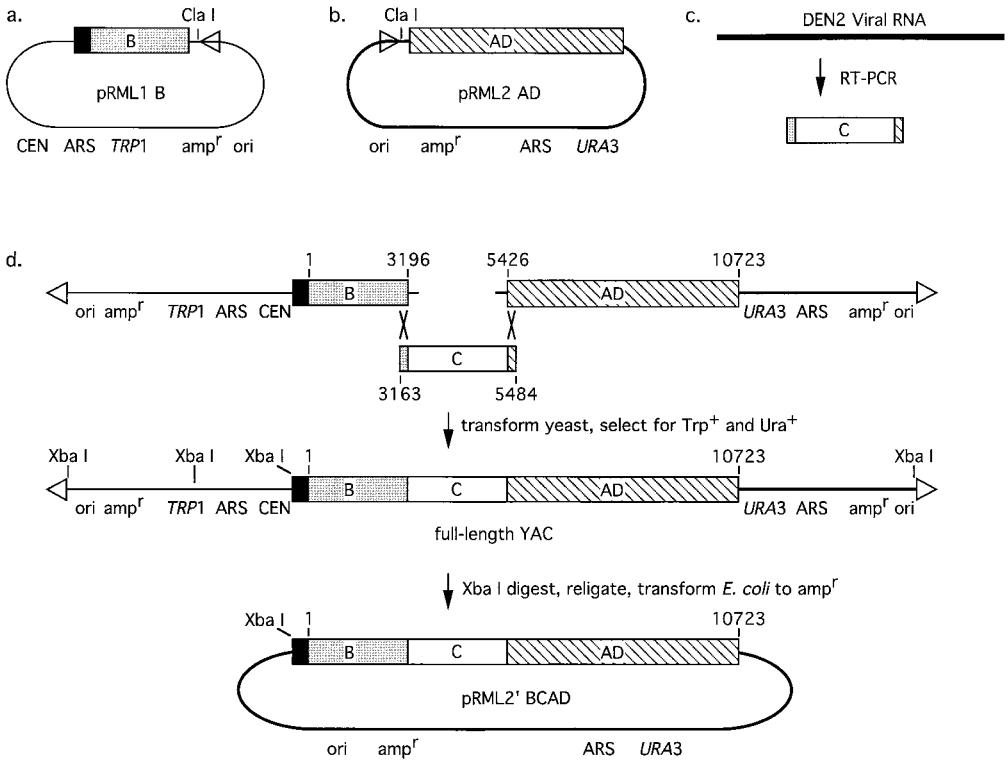

FIG. 1. Construction of a full-length DEN2 cDNA clone by using YAC technology. (a) Plasmid pRML1 B contains DEN cDNA B cloned into the polylinker of pRML1. The bacterial replication origin (ori) and selectable marker (amp^r), and the yeast replication origin (ARS), selectable marker (*TRP1*), and centromere (CEN) are indicated. A telomere (triangle) and the unique *Cla*I site are also shown. (b) Plasmid pRML2 AD contains DEN cDNAs A and D cloned into the polylinker of pRML2. The bacterial replication origin and selectable marker are indicated as above. The yeast replication origin (ARS) and selectable marker (*URA3*) and a telomere (triangle) near the unique *Cla*I site are also shown. (c) The cDNA product C was amplified directly from the DEN2 genome by RT-PCR, so that it had a 34-nt overlap with cDNA B and a 59-nt overlap with cDNA A. (d) *Cla*I-digested pRML1 B and pRML2 AD were mixed with DEN cDNA C, and the mixture was used to transform yeast strain YPH857 to Trp$^+$ and Ura$^+$. In yeast, the indicated double homologous recombination event generates a YAC containing the full-length DEN cDNA. Subsequently, DNA isolated from yeast harboring the full-length YAC was digested with *Xba*I, recircularized, and used to transform *E. coli* to ampicillin resistance, resulting in the clone pRML2′ BCAD, consisting of a modified version of pRML2 lacking the telomere, plus the full-length DEN cDNA. Crossover regions (X), pRML1 vector sequences (thin lines), pRML2 vector sequences (medium lines), the DEN2 RNA (thick line), and the SP6 RNA polymerase promoter (black box) are indicated. Various regions of DEN2 cDNA are also shown (other boxes). Pertinent restriction sites and DEN2 nucleotide numbers are indicated.

of cDNA C with termini which overlap cDNA B and cDNA A (Fig. 1c). This cDNA C product was mixed with *Cla*I-linearized pRML1 B and pRML2 AD, and the mixture was used to transform the *trp1 ura3* yeast strain YPH857 to Trp$^+$ Ura$^+$. In yeast, recombination between the short homologous regions at the termini of cDNA C with the B fragment in pRML1 B and the A fragment in pRML2 AD can generate a YAC containing a telomere at each end, both sets of yeast and *E. coli* origins and selectable markers, a centromere, and the full-length DEN2 NGC cDNA (Fig. 1d). Trp$^+$ Ura$^+$ yeast colonies were picked and directly screened by PCR for the presence of cDNA C, and 18 of 24 were positive (data not shown). DNA was prepared from cultures of 11 of these colonies, and the structure of the DEN sequences was analyzed by Southern blotting. This analysis showed that 8 of the 11 YACs contained full-length DEN2 cDNA (data not shown); for the other three, it appeared that the restriction digest failed, leaving them uncharacterized.

The strategy used to assemble full-length DEN2 cDNA in a yeast shuttle vector was similar to the YAC method and is illustrated in Fig. 2. First, pRS BAD was constructed in *E. coli* by cloning the DEN cDNA fragments B and A plus D into the polylinker of pRS424, oriented as shown in Fig. 2a. The vector pRS424 contains sequences for replication and selection in *E. coli* (ori and Amp^r) and in yeast (2μm and *TRP1*). The cDNA C fragment was made from DEN2 NGC RNA by RT-PCR (Fig. 2b) so that it overlapped both the B and the A fragments, as described above. Digestion of pRS BAD with *Xho*I resulted in a linear molecule (Fig. 2c). The cDNA C product was mixed with *Xho*I-linearized pRS BAD, and the mixture was used to transform yeast strain YPH857 to Trp$^+$ (Fig. 2c). Double homologous recombination in yeast between cDNA C and the linearized pRS BAD can repair the gap in the shuttle vector to produce a circular molecule containing the full-length DEN2 cDNA, referred to as pRS BCAD. Trp$^+$ colonies were screened directly by PCR for the C fragment, and 11 of 18 were positive (data not shown). Six of these colonies were grown, and DNA was prepared and analyzed by Southern blotting; five of the six had the pattern expected for full-length DEN2 cDNA, while the sixth appeared to have been a partial digest (data not shown).

Purification of full-length clone DNA. Initially, we attempted to purify pRS BCAD DNA directly from yeast. However, the yield of DNA was low, less than 0.5 μg of DNA from 250 ml of yeast culture. We also attempted to amplify the copy number of the full-length YAC (25) as a first step towards gel purification, but this too was unsuccessful.

Consequently, we decided to propagate the full-length clones in bacteria in order to obtain enough material to work with. For pRS BCAD, DNAs prepared from three independent yeast cultures were used to transform *E. coli* to ampicillin resistance. However, additional work was necessary to prepare

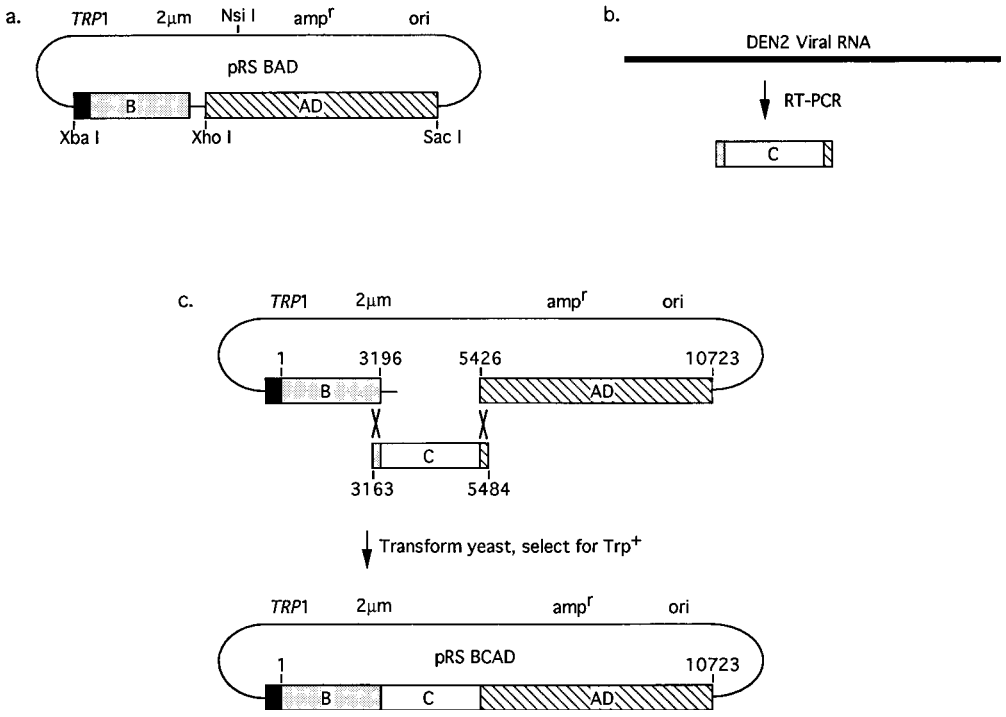

FIG. 2. Construction of a full-length DEN2 cDNA clone in the yeast shuttle vector pRS424. (a) DEN cDNAs B, A, and D were cloned into the polylinker of the shuttle vector pRS424 to make pRS BAD. The bacterial replication origin (ori) and selectable marker (amp^r) and the yeast 2μm replication origin and selectable marker (*TRP1*) are indicated. (b) cDNA fragment C was amplified directly from the DEN2 genome by RT-PCR so that it had short overlaps with the B and A fragments. (c) *Xho*I-linearized pRS BAD and cDNA C were mixed and used to transform yeast strain YPH857 to Trp$^+$. In yeast, recombination between the short homologous regions at the termini of cDNA C with the cDNA B and A fragments in pRS BAD generates the full-length cDNA, as shown. Crossover regions (X), vector sequences (thin lines), the DEN2 viral RNA (thick line), and SP6 RNA polymerase promoter (black box) are indicated. Various regions of DEN2 cDNA are also shown (other boxes). Some restriction sites used in the cloning and DEN2 nucleotide numbers are indicated.

the full-length YAC for transformation of bacteria (Fig. 1d). DNAs prepared from cultures of two independent yeast colonies harboring the full-length YAC were digested with *Xba*I and self-ligated at a low DNA concentration, to favor intramolecular ligation reactions. As can be seen in Fig. 1d, the desired product of this manipulation is pRML2′ BCAD, a modified version of pRML2 lacking the telomere but containing the full-length DEN2 cDNA. A portion of each self-ligation reaction mixture was used to transform *E. coli* to ampicillin resistance. For these transformations, as well as for the three involving pRS BCAD, *E. coli* STBL2 was used, since this strain has been designed to tolerate sequences that are unstable in other strains. Colonies were picked and usually were grown as 50-ml cultures, from which plasmid DNAs were prepared for analysis by restriction digestion.

Two size classes of *E. coli* colonies were obtained in each transformation. Because experience showed that the larger colonies almost always had an incorrect restriction pattern, smaller colonies were routinely picked. A total of 32 small colonies potentially containing pRS BCAD were screened; 28 had the correct restriction pattern, 2 were mixed right and wrong, and only 2 were completely wrong. For the YAC experiment, 26 colonies potentially harboring the full-length clone pRML2′ BCAD were screened; 24 had an incorrect restriction pattern and presumably harbored other possible products of the self-ligation reaction, and 2 had a pattern consisting of the right restriction fragments plus an extra *Xba*I fragment. In both cases, the extra fragment was located upstream of the SP6 promoter and thus would not interfere with transcription of the DEN2 sequences. The identity of the extra fragment in these two clones was not further investigated.

Full-length RNA transcripts are infectious. Full-length clone DNAs were linearized just beyond the 3′ end of the DEN2 sequences with *Sac*I or with its neoschizomer *Ecl*136II. Linearized DNAs served as templates for in vitro transcription with SP6 RNA polymerase in the presence of cap analog. Analysis of the transcription reaction products by agarose gel electrophoresis showed one homogeneous product RNA band which was resistant to DNase but sensitive to RNase, as expected (data not shown). These transcripts are expected to be nearly identical to DEN2 viral RNA but with one extra G residue at the 5′ end and either an extra G (for the *Sac*I-digested templates) or an extra GAG (for the *Ecl*136II-digested templates) at the 3′ end.

Transcription reaction mixtures were used to transfect LLC-MK_2 cells by use of Lipofectin or by electroporation. DEN2 viral RNA was used as a positive control. Cells were observed daily for the appearance of CPE; representative data are presented in Table 1. CPE arose more quickly following electroporation, and by either method, CPE appeared first in cells transfected with DEN2 viral RNA and was detected a few days later in cells transfected with full-length transcripts. The infectivity was resistant to DNase but sensitive to RNase, indicating that the RNA transcript was the infectious moiety. The infectivity was also dependent on the presence of cap analog in the transcription reaction mixture. The infectivity of transcripts was also demonstrated by indirect immunofluorescence (Fig. 3). In this experiment, positive fluorescence could be detected in some cells as soon as 1 day postelectroporation, the earliest time examined. The percentage of fluorescing cells increased progressively from 1 to 7 days postelectroporation, by which time essentially all cells in the dish were positive. In cells

TABLE 1. Infectivity of RNA following transfection or electroporation

Transfection method and RNA source	Days CPE was detected[a]
Lipofectin transfection	
Complete transcription reaction mixture	13–15
Without cap analog	None
With RNase	None
With DNase	13–15
DEN2 viral RNA	10–11
Electroporation	
Complete transcription reaction mixture	4–6
DEN2 viral RNA	2–4

[a] The number of days after transfection or electroporation of LLC-MK_2 cells typically required until the first appearance of CPE.

transfected by using Lipofectin, positive fluorescence was generally first observed a few days before CPE (data not shown).

Numerous experiments were performed to test the infectivity of a series of full-length clone DNAs prepared from *E. coli*. Of the 28 pRS BCAD DNAs with the correct restriction pattern, 20 were tested, as were the two DNAs with a mixed pattern. Overall, 60% of the clones (12 of 20) with the right restriction pattern were infectious. Infectious transcripts were made from *Ecl*136II-digested templates, indicating that the presence of up to 3 extra nt at the 3′ end of the transcripts did not abrogate infectivity. Of the two YAC-derived pRML2′ BCAD plasmids, only one was infectious.

A comparison of the specific infectivities of RNA transcribed from one full-length cDNA pRS BCAD clone (3-3) and DEN2 viral RNA was made. LLC-MK_2 cells were electroporated with dilutions of transcription reaction mixtures or viral RNA preparations containing between 0.5 and 50 ng of RNA (estimated by ethidium bromide staining of undiluted samples in agarose gels), plated into wells, and then overlaid as for a standard plaquing experiment. In four independent experiments, DEN2 viral RNA averaged 68 PFU/ng (range, 26 to 118 PFU/ng; SEM = 23 PFU/ng), and transcript RNA averaged 1.9 PFU/ng (range, 1.2 to 2.4 PFU/ng; SEM = 0.3 PFU/ng). The ratio of specific infectivity of viral RNA to specific infectivity of transcript averaged 37.5 (range, 12 to 82; SEM = 17.5).

Clarified supernatants from transfected cells showing CPE were used as inocula to passage the DEN infection to naive LLC-MK_2 cells. Infectivity of supernatants was resistant to prolonged RNase digestion, consistent with the production and release of DEN particles from transfected cells. After one or two passages in LLC-MK_2 cells, media were used to infect C6-36 cells to make high-titer stocks of transcript-derived viruses. Titers of $10^{5.7}$ to $10^{6.7}$ were typically obtained, similar to the titer of DEN2 NGC stocks grown on these cells. Transcript-derived viral plaques were indistinguishable from DEN2 NGC plaques.

An experiment was done to demonstrate the presence of a *Bst*EII site in viral RNA from transcript-derived virus-infected cells, in order to rule out the possibility of accidental cross-contamination with parental DEN2 NGC. RT-PCR was used to amplify a 3.2-kb cDNA fragment of DEN2 (nt 2250 to 5484) from intracellular RNAs prepared from cells infected with transcript-derived viruses or DEN2 NGC (Fig. 4a). This cDNA fragment spans the position in the genome (nt 3191) where the *Bst*EII site was introduced into the full-length clones. In cells infected with DEN2 NGC, the 3.2-kb cDNA fragment was resistant to *Bst*EII digestion, as expected, but in cells infected with any of three different transcript-derived viruses the 3.2-kb fragment was cut once by *Bst*EII, creating the expected fragments of 2.3 and 0.9 kb (Fig. 4b). This confirms that the RNA

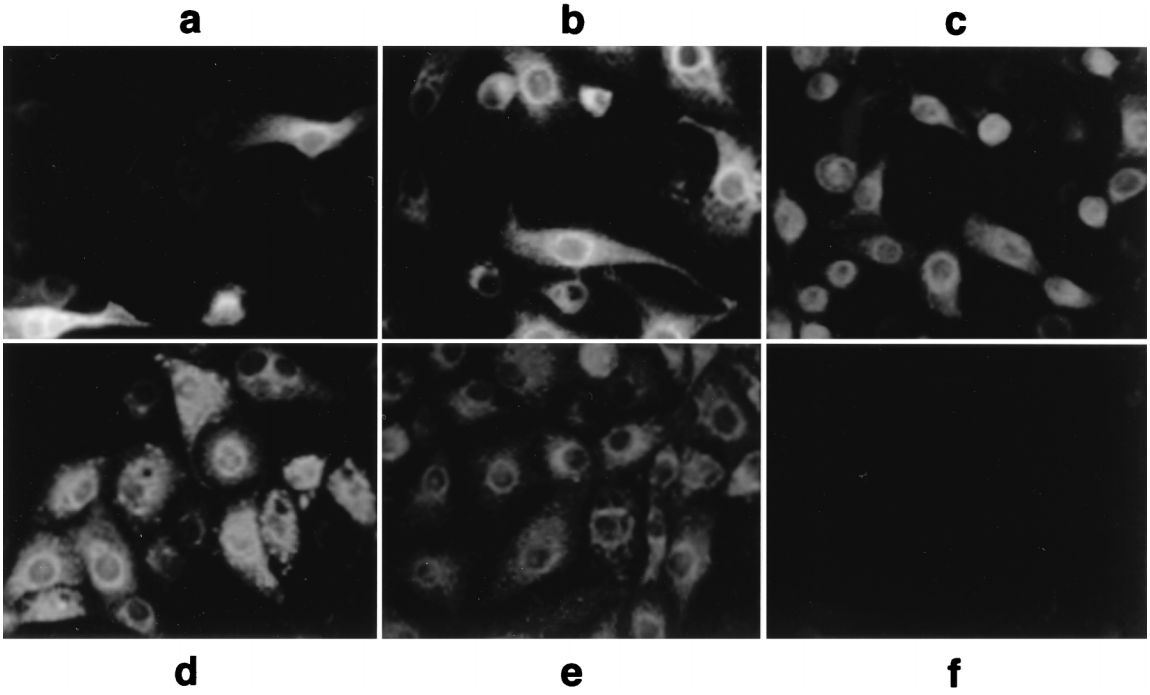

FIG. 3. Immunofluorescence analysis of cells electroporated with full-length RNA transcript. LLC-MK_2 cells were electroporated with transcripts from clone 3-3 or were mock electroporated as a control. DEN antigens were detected by indirect immunofluorescence at various times after electroporation. (a to e) Cells 1, 2, 3, 4, and 7 days, respectively, after electroporation with transcript. (f) Control cells 5 days after mock electroporation.

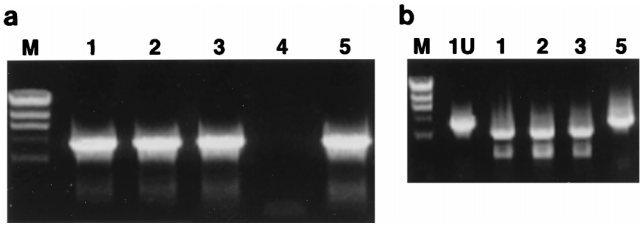

FIG. 4. Demonstration that RNA from cells infected with transcript-derived virus contained the introduced *Bst*EII site. C6-36 cells were mock infected or were infected with DEN2 NGC or with transcript-derived virus stock 3-3, 3-4, or 3-5. Upon observation of maximum CPE (usually 5 to 7 days postinfection), cells were harvested, and intracellular RNA was purified and subjected to RT-PCR to amplify a 3.2-kb DEN2 cDNA fragment (DEN2 nt 2250 to 5484). The resulting cDNA fragments were digested with *Bst*EII. cDNA made from DEN2 NGC-infected-cell RNA should not contain a *Bst*EII site, whereas cDNA made from transcript-derived virus infected-cell RNA should be cut once to produce fragments of 2.3 and 0.9 kb. (a) RT-PCR products: lane M, λ *Hin*dIII markers; lanes 1 to 3, cells infected by 3-3, 3-4, and 3-5, respectively; lane 4, uninfected cells; and lane 5, DEN2 NGC-infected cells. (b) *Bst*EII digestion of RT-PCR products: lane M, λ *Hin*dIII markers; lane 1U, undigested product from 3-3-infected cells; lanes 1 to 3, *Bst*EII-digested products from cells infected by 3-3, 3-4, and 3-5, respectively; lane 5, *Bst*EII-digested product from DEN2 NGC-infected cells.

transcripts were infectious and furthermore shows that the transcripts were replicated and packaged into the virus particles which were then used to passage the infection.

Biological characterization of transcript-derived virus in vitro. Transcript-derived virus stocks were compared with DEN2 NGC for growth on both LLC-MK_2 cells (Fig. 5a) and C6-36 cells (Fig. 5b). In these experiments, cells were infected at the same MOI with either DEN2 NGC or one of the transcript-derived virus stocks, samples of the media were removed daily, and the titers of virus were subsequently determined. The results show that transcript-derived viruses 3-3 and 5-2 grew at the same rate as the parental DEN2 NGC on LLC-MK_2 cells, and the same was true of virus 18-2 on C6-36 cells. The titer of virus 3-3 grown on C6-36 cells lagged slightly behind that of DEN2 NGC during the first few days after infection, but the final titers obtained for the two viruses were comparable. It is possible that virus 3-3 has a mild growth defect on C6-36 cells. However, since the initial slopes of the growth curves of virus 3-3 and DEN2 NGC were similar, it seems likely that the actual MOI of 3-3 was a little lower than that of DEN2 NGC in this experiment and that the 3-3 virus has no growth defect.

Next, DEN-specific protein expression in cells infected by transcript-derived virus was compared with that in cells infected by DEN2 NGC. Infected cells were radiolabeled with [^{35}S]methionine for 4 or 21 h, and cell extracts were immunoprecipitated with DEN2-specific HMAF and analyzed by SDS-PAGE and fluorography (Fig. 6). Each of the DEN2-specific NS2B, prM, NS1, E, and NS3 bands was identified on the basis of its mobility and resistance or sensitivity to endoglycosidases (data not shown). A DEN2-specific glycoprotein (indicated by an asterisk) appeared with varying intensity in different gels. We suspect that it may be dimeric NS1, apparently present in samples that were not completely heat denatured (29). The pattern of DEN-specific proteins observed for transcript-derived virus 3-3 was identical to that for DEN2 NGC under both labeling conditions. Taken together with the growth curve results, this suggests that the transcript-derived virus is biologically equivalent to wild-type DEN2 NGC in cells in culture.

Comparison of the sequences of an infectious clone and DEN2 NGC. To further characterize the infectious clones, the DNA sequence of the DEN-specific portion of one of the clones (pRS BCAD 3-3) was determined and compared with

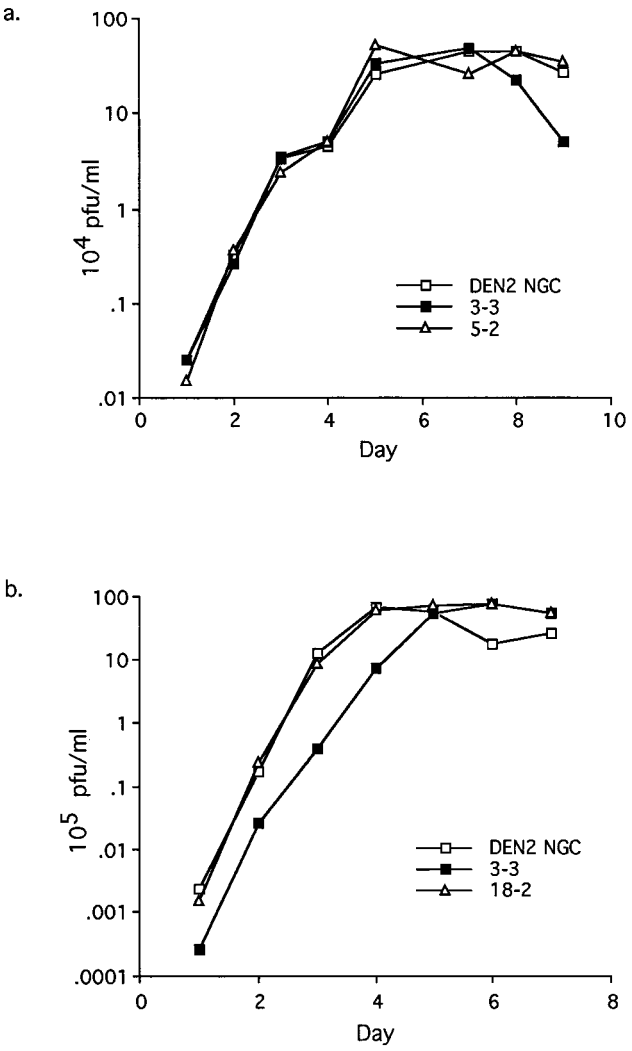

FIG. 5. Growth of transcript-derived viruses versus DEN2 NGC. LLC-MK_2 cells were infected at an MOI of 0.01 PFU/cell with DEN2 NGC or with transcript-derived virus stock 3-3 or 5-2 (a) or C6-36 cells were infected at an MOI of 0.0025 PFU/cell with DEN2 NGC or with transcript-derived virus stock 3-3 or 18-2 (b). A sample of the medium was removed daily, and the virus titer in each sample was determined on LLC-MK_2 cells.

the published DEN2 NGC sequence (14). As can be seen in Table 2, there were 16 locations in the genome where the infectious clone sequence differed from that published for DEN2 NGC. Only one of these, the silent C-to-T mutation at nt 3198 that introduces the *Bst*EII site, was expected. Of the 15 unexpected changes, 14 were in the open reading frame (8 silent and 6 missense) and 1 was in the 3′ noncoding region. After getting this result, we determined the sequence at these 15 locations in our laboratory DEN2 NGC strain by direct sequencing of RT-PCR products. At 13 of these 15 locations, the laboratory strain sequence agreed with that of pRS BCAD 3-3. At another position (nt 10195), the laboratory strain had an equal mixture of A and T, where the infectious clone had a T and the published DEN2 NGC sequence was an A. At only one of these 15 locations, the silent T-to-C mutation at nt 3744, did the laboratory strain sequence agree with the published sequence and differ from that of the clone. Thus, despite the use of RT-PCR as part of the cloning process, the final DNA sequence of the infectious clone appears to be a very accurate

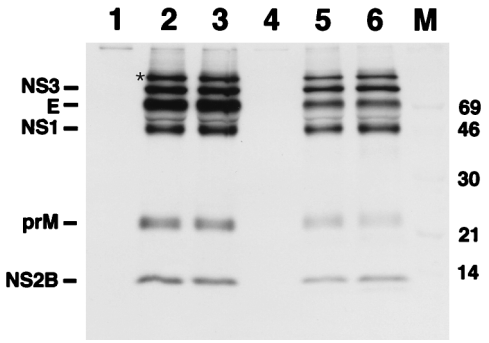

FIG. 6. Protein expression by transcript-derived virus. LLC-MK_2 cells were mock infected (lanes 1 and 4) or were infected at an MOI of 0.125 PFU/cell with transcript-derived virus stock 3-3 (lanes 2 and 5) or with parental DEN2 NGC (lanes 3 and 6). At 3 days postinfection, cells were labeled with [^{35}S]methionine for either 4 (lanes 1 to 3) or 21 (lanes 4 to 6) h. Cell lysates were immunoprecipitated with DEN2 HMAF, and the precipitates were analyzed by SDS-PAGE and fluorography. Lane M, ^{14}C-labeled protein markers, with molecular masses (in kilodaltons) indicated on the right. The positions of several DEN-specific bands are indicated on the left. *, unidentified DEN-specific band.

copy of the starting laboratory strain DEN2 NGC sequence: only two silent mutations, one of which had been introduced intentionally, occurred in a total of 10,723 nt.

DISCUSSION

This work describes the assembly of full-length cDNA of DEN2 NGC in yeast, either as part of a YAC or in a yeast-*E. coli* shuttle vector. After recovery of plasmids in *E. coli* STBL2, RNA transcripts were made in vitro and were shown to be infectious. The specific infectivity of transcripts was estimated as 2 PFU/ng, which was roughly 40-fold less than that of viral RNA. These numbers are comparable to what has been reported for other flavivirus infectious transcripts (18, 22, 28). There are numerous factors which might contribute to the reduced specific infectivity of transcripts, including the extra nucleotides at the ends, incomplete capping, premature termination (or failure of termination) of in vitro transcription, and inhibition of transfection by components of the transcription reaction mixture. The few extra nucleotides at the 3′ end of our DEN2 transcripts do not seem to have much of an effect, since RNA transcripts with 3 extra nt at the 3′ end (a consequence of *Ecl*136II linearization of templates) seemed as infectious as those with a single extra G (following *Sac*I linearization). We suspect that these extra nucleotides do not remain a part of the transcript-derived DEN2 virus genome, because it has been shown that extra nucleotides at the ends of Kunjin virus infectious transcripts were precisely removed (17). Transcript-derived DEN2 was equivalent to parental DEN2 NGC for growth and protein expression in tissue culture cells, and sequencing of one of the infectious clones confirmed that the cloned cDNA accurately reflects the sequence present in our laboratory strain of DEN2 NGC.

It is interesting that initial attempts to make this infectious clone in *E. coli* were unsuccessful but that once obtained in yeast, such clones could be grown in *E. coli* to produce working stocks of DNA. We do not know why this is true. One possibility is that the DEN sequences are simply more stable in the pRS424 and pRML2 vectors than in pGEM11Zf$^+$ or pCL1921, and the entire construction might have been done in bacteria by using these vectors. However, an attempt to make the full-length clone directly in *E. coli* by ligating the DEN2 cDNA C fragment into pRS BAD and transforming STBL2 cells failed. Furthermore, DNA isolated from the larger colonies on bacterial transformation plates almost always had the wrong restriction pattern, suggesting that cells containing rearranged plasmids have a growth advantage. Also, transformation of correct plasmids back into *E. coli* again results in two colony size classes, with large colonies containing rearranged DNA and small colonies containing correct DNA. All of this suggests that the full-length DEN2 sequences are not completely stable in the pRS424 or pRML2 vector. Similar instability problems have plagued all who have tried to make full-length flavivirus cDNA clones in *E. coli*. Many of the rearrangements involve

TABLE 2. Differences between the sequence of the infectious clone and the published DEN2 NGC sequence and comparison to the laboratory DEN2 NGC strain sequence

Sequence change no.	nt no.	Sequence			Gene region	Amino acid change
		Infectious clone[a]	Published DEN2[b]	Laboratory strain[c]		
1	1075	A	G	A	E	E to K
2	3198[d]	T	C	C	NS1	None (silent)
3	3744	C	T	T	NS2A	None (silent)
4	3876	C	T	C	NS2A	None (silent)
5	4428	T	C	T	NS2B	None (silent)
6	4932	T	G	T	NS3	None (silent)
7	6177	T	C	T	NS3	None (silent)
8	6317	G	A	G	NS3	K to R
9	6486	T	A	T	NS4A	None (silent)
10	8199	C	T	C	NS5	None (silent)
11	8230	G	T	G	NS5	L to V
12	8531 and 8532	TG	GT	TG	NS5	G to V
13	8583	G	A	G	NS5	None (silent)
14	10195	T	A	A and T	NS5	N to Y
15	10241	G	A	G	NS5	K to R
16	10414	A	T	A	3′ noncoding	N/A[e]

[a] The sequence of pRS BCAD 3-3 was determined.
[b] The published DEN2 NGC sequence is from Irie et al. (14).
[c] Sequence of select regions of RT-PCR products made from the laboratory DEN2 NGC strain.
[d] The C-to-T change at nt 3198 was intentionally introduced to create a *Bst*EII site.
[e] N/A, not applicable.

insertions of DNA into the flavivirus sequences, so it seems that the problem is not simply one of total insert size. Most characterized insertions and deletions in the DEN4 infectious clone mapped to the E/NS1/NS2A region (7), suggesting that *E. coli* does not tolerate this portion of the genome, perhaps because of a secondary structure or the adventitious expression of some toxic product.

In contrast to the situation in *E. coli*, the available evidence suggests that the full-length cDNA clone is probably stable in yeast. First of all, there were no observed rearrangements in the DEN sequences in any of the full-length constructs in yeast as assayed by Southern blotting. Further, infectious clones were recovered in *E. coli* from all three yeast colonies harboring pRS BCAD, suggesting that all shuttle vector constructs in yeast with the correct full-length DEN2 cDNA were potentially infectious. This is in sharp contrast with DNAs isolated from *E. coli*, where 8 of 20 DNAs with the correct restriction pattern were not infectious, presumably due to undetected aberrations in the DEN sequences. Given the apparent greater stability of the full-length clones in yeast than in bacteria, it may be preferable to grow large-scale yeast cultures for DNA production, especially in cases where the full-length cDNA is less stable in *E. coli* than these DEN2 NGC clones. It might also be possible to produce DEN2 transcripts directly in *Saccharomyces* by incorporating a ribozyme to generate the 3′ end (3) and either expressing SP6 RNA polymerase in yeast or replacing the SP6 promoter with a yeast promoter. It is even conceivable that DEN will replicate in yeast, as has been shown for another positive-strand virus, brome mosaic virus (15).

Of the two methods used to assemble the full-length DEN2 clone, the approach using the shuttle vector was simpler and worked more reliably than the YAC approach, due of course to the requirement for an extra cloning step before the infectious clone could be recovered from the YAC in bacteria. Even if infectious clone DNA were to be prepared directly from yeast, it seems likely that the shuttle vector would produce a better yield, especially if the YAC copy number cannot be amplified. Thus, all ongoing work with these infectious clones is being done with the shuttle vector; the YAC approach has been discontinued. With the shuttle vector, it has proven easy to introduce mutations into the DEN2 genome, using methods similar to those outlined in Fig. 2: mutations are engineered into cloned DNA fragments by standard methods and are then incorporated into the DEN genome by gap repair homologous recombination in yeast, followed by recovery of the mutated full-length clone in STBL2 cells. Studies are in progress to assess the effects of the introduced mutations on the phenotype of the virus.

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ADDENDUM IN PROOF

Recent experiments have shown that DEN2 virus can be recovered from cells electroporated with uncapped RNA transcripts. The data indicate that the specific infectivity of uncapped RNA is significantly reduced compared to that of capped RNA.

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